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6. AUTHOR(S)

Dr. Charles R. Cantor

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Dr. Charles R. Cantor
Center for Advanced Biotechnology
Boston University
36 Cummington St.
Boston, MA 02215

8. PERFORMING ORGANIZATION REPORT NUMBER

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U.S. Army Research Office
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13. ABSTRACT (*Maximum 200 words*)

This project had three specific aims. The first was the design, creation, and testing of genetically engineered microorganisms that degrade specific hazardous compunds and then self-destruct under natural environmental conditions. The second was to design and characterize novel self-assembly systems to generate small structural biomaterials for biological and materials science applications. The third was to develop sensitive monitoring systems for microorganisms usable in the field. In the first project, we successfully created a streptavidin-based suicide system. We also designed a supplementary, potentially very powerful suicide system and constructed part of it. In the second project, anti-parallel coiled-coil sequences have successfully been fused to the C-terminus of streptavidin. In the third project, green flourescent protein (GFP) has been fused to streptavidin, and the resulting fusion has shown potential promise for the GFP moiety to become a sensitive monitoring tag usable in the field.

14. SUBJECT TERMS

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1. FORWARD

The principal goal of this program was the development of improved suicide systems for controlling the viability of environmentally released genetically engineered microorganisms. The major accomplishment of the program is the establishment of such a system in the bacterium *Pseudomonas putida* which is able to metabolize aromatic hydrocarbons using enzymes coded for genes on the TOL plasmid. The suicide element is *streptavidin,* a protein produced by the bacterium *Streptomyces avidinii* that binds the essential vitamin d-biotin with such high affinity that the binding is effectively irreversible. The major trick developed to make the suicide system stable and affective is the creation of a two-level genetic switch. Control at both the protein level and the antisense RNA level was used to prevent any leakage in streptavidin production until hydrocarbon in the environment was depleted and the TOL expression became uninduced. In the other projects funded by the program novel self assembly systems were examined as possible structural materials for biological and other nanofabrication applications, and streptavidin-green fluorescent protein fusions were created as possible sensitive monitoring tags for gene expression in the field.

2. PROGRESS REPORT

B. Statement of the Problem

Genetic engineering is potentially a powerful way to produce modified microorganisms targeted for specific tastes. One area that interested us was the use of modified organisms for environmental remediation. A second area of interest is

organisms modified to product unique materials. One principal hurdle in both of these applications is developing genetic designs that have industrial strength.

Release of a genetically modified organism for environmental credation has the potential risk that chemical contamination of the environment is simply replaced by biological contamination. To circumvent this problem it is desirable to have a controlled suicide system that eliminates the released organism once the remediation is complete. Controlled suicide can be achieved in principle by relatively simple genetic constraints in which the catabolic pathway and suicide pathway are coupled vis an either-or regulatory network. The difficulty is that the control has to be virtually perfect. Any leakiness of the suicide element while catabolism is active will lead to a survival disadvantage for the organism. Hence a selection scheme is established for organisms in which the suicide pathway is disabled by the accumulation of mutations. The effect of this is catastrophic. The more complex the suicide pathway the more targets of opportunity there are that can inactivate it. Tight control, alone, does not solve the problem. A severely repressed suicide pathway is likely to induce very slowly. During this sub-lethal induction period, organisms that have begun to express the pathway are at a selective disadvantage for growth. Hence any organisms that acquire mutations that disable the pathway take over and escape suicide. This is not an easy problem to overcome.

Production of genetically engineered novel materials faces a different challenge. Frankly stated, most biological materials are fragile and fail. They are not suited to industrial manufacturing standards. Only a small evident of natural biological materials are environmentally robust. Most of these materials like spider web proteins or silk filaments are different to handle to by existing methods of recombinant DNA technology. The challenge is to find an environmentally stable protein that is easy to

manipulate generically and then convent it into products with useful materials science applications.

C. Summary of Most Important Results

The main thread in the two projects supported by this program is the protein streptavidin. Produced by the bacterum *Streptomces avidinii,* streptavidin is a marvel of nature. It is a potent natural antibiotic. *S. avidinii* secretes it in an inactive form and then secretes a protease that activates it. The mature protein binds the natural vitamin H (d-biotin) with such avidity that organisms in the neighborhood of *S. avidinii* perish by starvation. It is a brilliant example of natural biological warfare. Since streptavidin must function extracellularly, it is not surprising that the protein is stable but the extent of the stability is a marvel. Streptavidin is arguably the most stable glubular protein known. It is resistant to proteases under almost all conditions, even the notorious proteinase K. The thermal denaturaion temperatures of biotin-saturated streptavidin is over 120° C, a temperature rarely encountered in nature. Thus once streptavidin has sequestered biotin, that biotin is gone for keeps.

We have exploited the unique properties of streptavidin as a suicide pill for environmentally released microorganisms. Here by selective production of streptavidin intracellularly we use its toxicity as a bacteriocide, not just a bacteriostatic agent. We have also begun to exploit streptavidin as a framework for genetically engineered materials. Here it is the stability and tetrameric nature of the protein that allows it to serve as a building block for multivalent constructs.

We first cloned the gene of steptavidin from as *S. avidinii* library expressed in *Escherichia coli* in 1985. Blind luck prevailed: our vector had no expression at all, so the gene for the active protein was correct. Others at the same time cloned inactive

mutants. Despite major efforts, all subsequent attempts to express the protein in *E. coli* failed, because of its toxicity until 1989 (?) when Takeshi Sano began to exploit the extremely tight control afforded by Studier's T7 expression system. Here a toxic gene is placed under the control of a bacteriophage T7 promoter, a promoter unrecognized by *E.coli* RNA polymerase but efficiently recognized by T7 polymerase. The latter is cloned under the influence of an inducible promoter but simultaneously T7 lysozyme, a protein inhibitor of T7 RNA polymerase is expressed under the control of a constitutive promoter. This buffers the system so that leakage that leads to low level T7 RNA polymerase expression has no phenotypic consequence.

In *E. coli* the T7 system works superbly well. Thus in the early stage of the project we patiently moved it, step by step, into *Pseudomonas putida,* and demonstrated functionality of each component. *P. putida* is a model organism for potential environmental remediation. It is a natural soil bacterium and carries on the TOL plasmid a complex catabolic operon capable of metabolizing a variety of aromatic hydrocarbons. Express of this operon is controlled by the product of the *Xyl S* gene which acts as a positive regulator in the presence of ligands like hydroxybenzoic acid (check!)

When we cloned the gene for streptavidin in *E. coli,* under the control of the T7 system, tight regulation resulted and no leakage was observed. When this entire system was moved to *P. putida,* the results were not as favorable. The system was leakier and an unacceptable rate of mutations that disabled the streptavidin expression cassette was observed. The T7 system as originally envisioned contained regulatory elements that operated only at the protein level as described earlier. To beef up this system with *P. putida* we conceived the notion of adding antisence RNA level control to the protein level control. The notion is simple but it proved to be very effective.

Elements that we wanted to be expressed out of phase were cloned head to head in the absence of transcription terminators. Thus, when one gene was actively transcribed it also expressed an antisense transcript to inhibit expression of the counterpart gene. When the constuct switched, the counterpart made antisense to ensure that the original gene was kept silent. This simple mechanism proved to be extremely effective, and it should be able to sense as a general model of the biological switch. The overall constructs are rather complex as shown in *Figure 1* but they function extremely effectively.

The suicide pill we have created in *P. Putida* has killing efficiency of 3 to 3.5 logs. This is ten fold better than the best system previously described, but it is not good enough to allow actual field use. In the final stages of the project we began to implement a second suicide system to complement the first one. The design requirements of the second system were that no components be in common with the first one. This is essential; otherwise a single mutation in the common element would intactivate both systems. The design of the second system is shown in *Figure.* 2. Unfortunately there was not sufficient time in the funded project to fully implement and test this design. In principle the second system ought to be even more effective than the first, since, upon consumption of aromatic hydrocarbons in the environment the *P. putida* will shut down a key gene responsible for the expression of a wide variety of often vital genes.

The second general project supported by this grant centered on preparing a set of two complementary streptavidins, designed as a framework for novel biomaterials. The plan was to prepare two streptavidin fusion proteins, conjugated with components of leucine zippers. In one case the zipper would extend from the streptavidin tetrameric core in an N to C direction. The second conjugate would have a leucine

zipper extending in the C to N direction from the streptavidin. Since leucine zippers form from antiparallel strants, we reasoned that each conjugate alone would be a stable, soluble tetramer but a mixture of the two should spontaneously aggregate through the formation of leucine zippers to form a stable ordered open network of streptavidin tetromers linked in four directions to others by leucine zippers. The biotin binding sites would still be free to allow doping of the network with biotinylated ligands, to create a variety of materials with novel properties. We were successful in creating and expressing the gene for one of the constructs, a leucine zipper fused to the C terminal of streptavidin. However, the complementary conjugate with a leucine zipper domain fused to the N terminus of streptavidin proved difficult to express and re-fold. Hence we were never able to prepare the desired aggregated complex.

2. Inventions

A. PUBLICATIONS AND TECHNICAL REPORTS

B. SCIENTIFIC PERSONNEL

Safanski
Takashi Sano
Cassandra L. Smith
Charlene Mello
David Kaplan